Classifying Melanocytic Tumors Based on DNA Copy Number Changes

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Melanoma and benign melanocytic nevi can overlap significantly in their histopathological presentation and misdiagnoses are common. To determine whether genetic criteria can be of diagnostic help we determined DNA copy number changes in 186 melanocytic tumors (132 melanomas and 54 benign nevi) using comparative genomic hybridization. We found highly significant differences between melanomas and nevi. Whereas 127 (96.2%) of the melanomas had some form of chromosomal aberration, only 7 (13.0%) of the benign nevi cases had aberrations. All seven cases with aberrations were Spitz nevi, in six of which the aberration was an isolated gain involving the entire short arm of chromosome 11. This aberration was not observed in any of the 132 melanomas. We also analyzed the 132 melanomas for genetic differences depending on anatomical site, Clark's histogenetic type, and sun-exposure pattern. We show that melanomas on acral sites have significantly more aberrations involving chromosomes 5p, 11q, 12q, and 15, as well as focused gene amplifications. Melanomas classified as lentigo maligna melanomas or as occurring on severely sun-damaged skin showed markedly more frequent losses of chromosomes 17p and 13q. This study shows a pattern of chromosomal aberration in melanoma that is distinct from melanocytic nevi and should be further evaluated as a diagnostic test for melanocytic lesions that are now ambiguous. In addition, we show marked differences in the genetic make-up of melanomas that depend on anatomical location and sun-exposure pattern indicating that potential therapeutic targets might vary among melanoma types. (*Am J Pathol* 2003, 163:1765–1770)

Melanocytes can give rise to a variety of benign and malignant neoplasms that differ in their clinical and histopathological appearance and more importantly in their biological behavior. The benign melanocytic tumors are called melanocytic nevi, and the malignant tumors are

called melanoma. Histopathology is the current gold standard for the diagnostic classification of melanocytic neoplasms. However, whereas histopathological criteria permit diagnosing the majority of melanocytic tumors as either benign nevi or melanoma, well-documented uncertainty exists in a significant number of cases.^{1–3} Misdiagnosis is relatively common and can result in inappropriate over or under treatment of patients. Together with lymphoma misdiagnosis of melanocytic lesions head the list of malpractice cases.^{4,5} Previous studies indicated differences in the patterns of chromosomal aberrations between melanomas and benign nevi that might be diagnostically helpful for ambiguous cases.^{6,7}

In addition to problems in classifying melanocytic lesions as benign or malignant there is controversy about whether melanoma is one homogenous disease or consists of biologically and histologically distinct subtypes. It has long been noted that melanomas can vary significantly in their clinical and histological presentation. Ocular melanoma shows a stereotypic pattern of metastasis to the liver and frequent aberrations of chromosome 3 separating it from cutaneous melanoma. Among melanomas affecting the skin there is marked variation of histopathology and clinical characteristics, partially depending on anatomical site and sun-exposure patterns. A classification of melanomas into several histogenetic types has been proposed.⁸ However, it is currently controversial whether the clinicopathological variations among cutaneous melanomas are reflecting true biological differences or whether they are secondary effects that depend on the skin architecture of different anatomical sites.⁹ A previous study indicated that among cutaneous melanomas, those that are located on acral skin (palms, soles, and subungual sites) differ from other types by a uniquely high frequency of focused amplifications.¹⁰

Genetic approaches have been of significant help to classify hematopoietic neoplasias¹¹ and as illustrated by recent advances in the treatment of chronic myeloid leukemia the identification of distinct aberrations followed by the identification of the precise genetic defect has paved the way to specific treatments.¹²

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We analyzed 186 melanocytic tumors (132 melanomas and 54 benign nevi) with comparative genomic hybridization (CGH) to determine aberrations that can help to separate benign melanocytic nevi and melanoma. We also explored whether there are genetic differences between melanomas depending on anatomical site, sun-exposure pattern, and Clark's histogenetic type.

Materials and Methods

Paraffin-embedded primary invasive melanomas were retrieved from the archives of the Dermatopathology Section of the Department of Dermatology, University of California, San Francisco, and in part from the Department of Dermatology, University of Würzburg, Würzburg, Germany. To be eligible for CGH analysis, cases were required to have at least one area from which a rather pure population of tumor cells could be isolated to yield sufficient amounts of DNA for CGH analysis.

A total of 186 cases were included in the analysis. Of these, 132 were histologically diagnosed as malignant and 54 as benign. The benign group consisted of 27 Spitz nevi, 19 blue nevi, and 7 congenital nevi. The CGH findings of 79 cases have been reported previously.^{6,7,10} The study was approved by the Institutional Review Board of the University of California, San Francisco.

DNA Extraction

Tumor-bearing tissue was microdissected from 30- μ m sections (2 to 20 per tumor) using hematoxylin and eosin-stained sections as guidance. DNA extraction and labeling was performed as published by Isola and colleagues.¹³ The amount of DNA obtained ranged from 2 to 12 μ g per specimen.

CGH

All measurements were performed in duplicate: once with 1 μ g of tumor DNA labeled with fluorescein-12-dUTP (Dupont Inc., Boston, MA), and 200 ng of Texas Red-5-dUTP-labeled reference DNA from normal human lymphocytes (standard labeling), and a second time with the labeling reversed as described previously.⁶ Normal DNA and DNA from tumor cell lines with known aberrations were used as negative and positive controls for CGH, respectively. CGH results were interpreted blinded to the histopathological information. We regarded a region as aberrant when either the standard labeling or the reverse labeling resulted in tumor:reference fluorescent ratios <0.80 or >1.2 or both the standard and the reverse labeling resulted in tumor:reference fluorescent ratios <0.85 or >1.15 .⁶ The former were termed "losses" and the latter were termed "gains." Based on CGH measurements, certain gained regions were additionally termed as "amplifications" if the tumor:reference ratio of a distinct segment of a chromosomal arm exceeded 1.5 or if the ratio elevation involved a sharply demarcated segment of a chromosomal arm. For the statistical analyses the data

were separated into 571 bins corresponding to the chromosomal bands of all autosomes.

Statistical Analysis

We compared the proportions of samples with aberrations in melanomas and nevi using the Fisher's exact test. Differences in the distribution of ages were tested using the two-sample *t*-test, whereas differences in gender proportions were tested using the binomial test. For these tests a *P* value of less than 0.05 was considered to indicate statistical significance.

We used the false discovery rate procedure of Hochberg and Benjamini to correct for multiple comparison.¹⁴ Let $H_1, \dots, H_{571}$ represent the null hypotheses of no change for each bin. Using this method, the *P* values from each bin are sorted from smallest to largest, denoted by $P_{[1]}, \dots, P_{[571]}$. All of the null hypotheses are rejected for which $P_i \leq P_{[i_a]}$, where i_a is the maximum index of *i* such that $P_{[i]} \leq \frac{i}{571}\alpha$. We fix α at 0.05. In addition, all bins with *P* values less than 0.01, unadjusted for multiplicity, are being reported.

Results

There was a highly significant difference in the frequencies and types of aberrations between melanomas and nevi (Figure 1). Whereas 127 (96.2%) of the melanomas had some form of aberration (mean number of aberrations, 7.5), only 7 of the nevi (13.0%) showed any aberrations ($P < 0.00001$). These seven nevi were all Spitz nevi and had only a single aberration each. This aberration was an isolated gain involving the entire short arm of chromosome 11 in six cases; the remaining Spitz nevus had an isolated gain of the distal chromosome 7. Remarkably, the gain involving the entire chromosome 11p was not found in any of the 132 melanomas. Copy number increases on parts of 11p were only seen in one melanoma in which there was an amplification involving the tip of chromosome 11p (11p15). Follow-up of Spitz nevi with chromosomal aberrations for up to 7 years did not reveal any indication of progression to melanoma.

Five melanomas (3.8%) showed no aberrations by CGH (Table 1). Two of these cases showed a significant admixture of lymphocytes, which may have led to a false-negative result. However, in the remaining three melanomas no technical explanation that could have obscured aberrations could be found. Two of these cases later showed regional metastasis.

The most frequently gained regions in the melanomas were 6p (37.1%), 1q (32.6%), 7p (31.8%), 7q (31.8%), 8q (25.0%), 17q (24.2%), and 20q (22.0%) (Figure 1). The most frequent losses were of chromosomes 9p (64.4%), 9q (36.4%), 10q (36.4%), 10p (29.5%), 6q (25.8%), and 11q (21.2%).

We analyzed the melanoma cohort for differences between clinicopathological subsets using three different comparisons. We compared the 22 melanomas that occurred on acral skin (palm, soles, or subungual sites) with

Figure 1. Copy number differences in melanoma (A) and melanocytic nevi (B). The y axes shows the proportion of copy number increases (green) and decreases lost (red) by chromosomal bin.

the 108 melanomas on nonacral skin (any other cutaneous site, except palm, soles, or subungual sites). We call the first group AMs and the second group non-AMs. AMs

had significantly more frequent copy number increases of chromosomes 5p (36.4% versus 4.6%, $P = 0.00015$) and 12q (22.7% versus 0%, $P = 0.000092$) than non-AMs. AMs also had markedly higher number of increases on 4q (18.2% versus 0.9%, $P = 0.0076$) and 11q (36.4% versus 9.3%, $P = 0.0028$), as well as losses involving chromosomes 6q (50% versus 19.4%, $P = 0.0052$), 15q (22.7% versus 0.9%, $P = 0.00049$) and 16q (36.4% versus 10.2%, $P = 0.0044$). The latter differences, however, did not reach significance when adjusted for multiple testing. The DNA copy number increases involving chromosomes 5p, 12q, and 11q were mostly caused by focused amplifications (see Materials and Methods for definition). Overall, there were strong differences in the frequencies of amplifications between AMs and non-AMs.¹⁰ Whereas all AMs (100%) had at least one amplification (mean, 1.91), amplifications were significantly less frequent in the nonacral melanomas (22.2%) ($P < 0.00001$). When the 22 melanomas on acral skin were analyzed by histological subtypes, 15 were categorized as acral lentiginous melanoma (ALM), 2 as superficial spreading melanomas (SSMs), 1 had overlapping features between ALM and SSM, and 4 were unclassifiable. Independent of their growth patterns all 22 cases showed amplifications.

The melanomas that were localized on nonacral skin ($n = 108$) were further divided using two different approaches. First, we compared SSMs with lentigo maligna melanomas (LMMs) using the histological definitions described by Clark and colleagues.⁸ Using these criteria 52 were categorized as SSM and 25 as LMM. Some aberrations occurred with markedly different frequencies between the two groups (Figure 2C). LMMs had more frequent gains on 15q (20.0% versus 0%, $P = 0.0027$) and 17q (40% versus 11.5%, $P = 0.0066$) and more frequent losses on 13q (32.0% versus 5.8%, $P = 0.0040$) and 17p (36.0% versus 3.8%, $P = 0.00043$). However, none of these aberrations reached significance when corrected for multiple testing.

The clinical and histopathological presentation of melanoma has been noted to vary depending on whether the skin has been exposed in an acute-intermittent pattern

Table 1. Summary Statistics of Age, Gender, Total Number and Type of Aberrations for Overall Cases, and the Subgroups that Were Compared

Variable	Overall ($n = 186$)	Malignant ($n = 132$)	Benign ($n = 54$)	AM ($n = 22$)	Non-AM ($n = 108$)	SSM ($n = 52$)	LMM ($n = 25$)	CSD ($n = 32$)	No-CSD ($n = 76$)
Age (SD)	53.7 (26.4)	68.0 (16.9)	22.5 (16.4)	59 (23.9)	48.9 (28.6)	44.7 (29.0)	55.2 (29.0)	59.1 (26.8)	44.4 (28.4)
Males/females [%]	52.2/47.8	50.8/49.2	55.6/44.4	52.4/47.6	49.5/50.5	46.2/53.8	60/40	56.3/43.7	46.7/53.3
Mean tumor thickness in mm (SD)	4.30 (3.82)	4.30 (3.82)	NA	5.83 (6.08)	3.97 (3.12)	3.60 (3.11)	3.74 (2.55)	3.55 (2.39)	4.16 (3.40)
Number of aberrations (range)	4.66 [0–20]	6.51 [0–20]	0.13 [0–1]	10.09 [4–17]	5.77 [0–20]	4.73 [0–13]	8.4 [0–20]	8.00 [0–20]	4.83 [0–13]
Number of gains (range)	2.31 [0–11]	3.21 [0–11]	0.13 [0–1]	5.50 [3–7]	2.73 [0–11]	2.29 [0–6]	3.92 [0–11]	3.88 [0–11]	2.25 [0–7]
Number of losses (range)	2.34 [0–10]	3.30 [0–10]	0 [0–0]	4.59 [0–10]	3.04 [0–10]	2.44 [0–7]	4.48 [0–10]	4.13 [0–10]	2.58 [0–7]
Number of amplifications (range)	0.39 [0–3]	0.52 [0–3]	0 [0–0]	1.91 [1–3]	0.24 [0–2]	0.23 [0–2]	0.28 [0–1]	0.44 [0–1]	0.16 [0–2]
Percentage with at least one aberration	72	96.2	13	100	95.4	92.3	96	96.9	94.7
Percentage with at least one gain	66.1	87.9	13	100	85.2	80.8	88	90.6	82.9
Percentage with at least one loss	66.1	93.2	0	95.5	92.6	90.4	96	93.8	92.1
Percentage with at least one amplification	27.4	34.8	0	100	22.2	19.2	28	43.8	13.2

Figure 2. Comparison of the aberration frequencies for each genomic locus between different types of melanomas. **A:** Frequencies of gains (up) and losses (down) of melanomas on acral skin (green) *versus* nonacral skin (red). **B:** Melanomas on chronically sun-damaged skin (green) *versus* not chronically sun-damaged skin (red). **C:** LMMs (green) *versus* SSMs (red). The **asterisk** indicates regions that occurred with marked difference ($P < 0.001$) between the groups. The **red asterisks** were significant when corrected for multiple testing.

versus a chronic pattern to UV light. To determine aberrations possibly associated with sun-exposure pattern, we analyzed the non-AM group for the presence of marked solar elastosis evidenced by a blue-grayish discoloration of the dermis as a proxy for chronic sun damage (CSD).¹⁵ Thirty-two non-AM cases showed marked solar elastosis and 76 did not show marked solar elastosis. When we compared the CSD group with the no-CSD group (Figure 2B) we found very similar results as in the comparison of LMMs and SSMs. The CSD group had more frequent gains involving chromosome 15q (12.5% *versus* 0%, $P = 0.0067$) and more frequent losses, unadjusted involving chromosome 17p (28.1% *versus* 6.6%, $P = 0.0044$). Similar to the LMM *versus* SSM comparison these findings were not significant when adjusted for multiple testing. The no-CSD group showed markedly higher losses, of chromosomes 10p (36.8% *versus* 9.4%, $P = 0.0046$) and 10q (40.8% *versus* 12.5%, $P = 0.0037$), but these differences also did not reach significance after correction for multiple testing. There were more frequent amplifications in the CSD group than in the no-CSD group (43.8% *versus* 13.2%, $P = 0.00046$). The most frequent region amplified in all groups was chromosome 11q13 (AM, 36.4%; LMM, 25.0%; SSM, 9.6%; CSD, 18.8%; no-CSD, 6.6%).

Discussion

Our data show significant differences in the aberration pattern of different types of melanocytic neoplasms. The most pronounced differences were observed between nevi and melanomas in which we observed two distinct patterns with very little overlap. Whereas the vast majority of melanomas (96.2%) had at least one aberration, most benign nevi did not have any aberrations. Only a subset of Spitz nevi showed aberrations. With the exception of one case these aberrations were restricted to copy number increase of the entire short arm of chromosome 11 as previously reported.⁷ Remarkably, this aberration was not seen in any of the 132 melanomas. The clear-cut differences between nevi and melanomas may indicate that gross chromosomal aberrations are an essential feature of most melanomas. Telomeric crisis is one of several possible mechanisms how chromosomal aberrations in

melanoma could arise. A role for telomeric crisis is supported by the observation that invasive primary melanomas have activated telomerase, whereas melanocytic nevi have not.¹⁶ It is attractive to speculate that melanocytic nevi stop proliferation and undergo replicative senescence once their telomeres shorten to a certain level whereas melanomas do not.¹⁷ Progressive telomere attrition with end-to-end fusion of chromatids during crisis could explain the frequent aberrations in melanoma. A role for replicative senescence in nevi is suggested by the association between nevus size and age at which the nevus is acquired. Only nevi acquired *in utero* can become very large in size, whereas nevi acquired after birth never reach sizes more than a few centimeters.

The fact that there is very little overlap in the aberration patterns between melanomas and nevi indicates that the detection of these DNA copy number changes is a helpful diagnostic tool. However, as such a tool would be applied to melanocytic tumors that are histologically ambiguous, and not clearly benign or malignant as in this study, the versatility of such a test needs to be validated in a separate study using cases that cannot be classified with current histological criteria.

The significant number of melanomas in this study also allowed us to compare aberration patterns of melanomas of different types. It has long been noted that the presentation of melanoma can vary significantly and a classification was proposed to separate melanoma into SSM, LMM, nodular melanoma (NM),¹⁸ and ALM.¹⁹ The criteria proposed for this classification were based on the growth pattern of the radial growth phase of a melanoma: a so-called pagetoid pattern with solitary and nested melanocytes scattered throughout the epidermis for SSM, as opposed to a lentiginous pattern with single melanocytes arrayed along the dermo-epidermal junction for LMM and ALM. NM was defined as an invasive melanoma without any notable radial growth. These histological patterns show some correlation with anatomical location (SSM and NM on trunk and extremities; LMM on the face; and ALM on the palms, soles, and subungual sites), sun-exposure patterns (acute-intermittent in SSM and NM, chronic in LMM, and no association in ALM), and patient age (SSM and NM more frequent in patients younger than 60 years, LMM and ALM more frequent in patients older than 60

Table 2. Aberrations that Showed the Most Differences in Frequencies in Comparisons Between Groups

Position	Aberration type	Groups compared		P value	Significance
12q14	Gain	AM; 5 (22.7%)	Non-AM; 0 (0%)	0.000092	Y
5pter	Gain	AM; 8 (36.4%)	Non-AM; 5 (4.6%)	0.00015	Y
17pter	Loss	LMM; 9 (36%)	SSM; 2 (3.8%)	0.00043	N
15q13	Loss	AM; 5 (22.7%)	Non-AM; 1 (0.9%)	0.00049	N
15q21.1	Gain	LMM; 5 (20%)	SSM; 0 (0%)	0.0027	N
11q13.3	Gain	AM; 5 (22.7%)	Non-AM; 10 (9.3%)	0.0028	N
10q22.2	Loss	no-CSD; 31 (40.8%)	CSD; 4 (12.5%)	0.0037	N
13q21.1	Loss	LMM; 8 (32%)	SSM; 3 (5.8%)	0.004	N
16q24	Loss	AM; 8 (36.4%)	Non-AM; 11 (10.2%)	0.0044	N
17pter	Loss	CSD; 9 (28.1%)	no-CSD; 5 (6.6%)	0.0044	N
10pter	Loss	no-CSD; 28 (36.8%)	CSD; 3 (9.4)	0.0046	N
6q23.1	Loss	AM; 11 (50%)	Non-AM; 21 (19.4%)	0.0052	N
17qter	Gain	LMM; 10 (40%)	SSM; 6 (11.5%)	0.0066	N
15q15	Gain	CSD; 4 (12.5%)	no-CSD; 0 (0%)	0.0067	N
4q11	Gain	AM; 4 (18.2%)	Non-AM; 1 (0.9%)	0.0076	N

The first column shows the chromosome band, followed by the type of aberration. The third and fourth columns the group with the higher or lower frequency of aberrations, respectively, followed by the number of cases with the aberration in this group and the percentage in parentheses. The last column indicates whether the *P* value in the second last column is significant (Y) after correction for multiple comparisons or not (N).

years). However, these associations overlap significantly and it is currently unclear, whether they truly reflect biologically distinct types of cutaneous melanomas, and if so what defines them. This problem is illustrated by the fact that the histological patterns of SSM and NM can also occur in melanomas evolving on palms and soles,²⁰ and the histological pattern of ALM has been reported for melanomas on acral skin outside of the palms and soles such as the dorsa of hands and feet.²¹

Our finding that melanomas on the soles, palms, and subungual skin invariably show gene amplifications independent of the microscopic growth pattern, and that amplifications occur only in a minority of melanomas on the remainder of the skin, suggests that anatomical location, ie, location on glabrous (nonhair-bearing) skin, not histological pattern, defines a distinct melanoma type. Therefore, we suggest using the term acral melanoma (AM) instead of ALM.²² AMs also showed certain aberrations that occurred with significantly different frequencies when compared to melanomas on nonglabrous skin. Copy number increases at the regions of cyclin D1, CDK4, and the platelet-derived growth factor receptor- α occurred significantly more frequent in this group (Table 2, Figure 2A), possibly indicating novel therapeutic targets.^{23,24} The distinctive type of genomic instability suggests that these melanomas may have a unique pathogenesis and should be evaluated separately in therapeutical trials.

Our analysis showed several regions with notably different aberration frequencies between SSM and LMM, although none of them remained significant when a rigorous correction for multiple comparisons was applied. However, it has to be noted that these differences are highly significant when regarded individually. Although, rigorous adjustment for multiple testing decreases the number of false-positive association it also increases the likelihood to miss associations that are true. Focused studies are needed to further evaluate these differences. It is notable that we found similar differences when the nonacral melanomas were stratified according to the presence or absence of marked solar elastosis instead of

histopathological pattern. This suggests that not the growth pattern but the occurrence of chronically sun-damaged skin may be the common denominator of these groups of melanomas. The regions that were more frequently aberrant in LMMs and CSDs included chromosome 13q harboring the nucleotide excision repair gene ERCC5, and the 17p spanning the location of P53. ERCC5 is one of several repair genes mutated in xeroderma pigmentosum, a condition that goes along with an increased risk for melanoma, predominantly of the LMM type.²⁵ P53 mutations have been previously reported in melanomas on sun-exposed skin²⁶ and others have found that LMMs have lower levels of p53 protein expression than SSMs.^{27–29} The LMMs/CSD cases in our series also had markedly lower p53 expression levels by immunohistochemistry (data not shown).

In summary, our study shows a recurrent pattern of chromosomal aberration in melanoma that is distinct from melanocytic nevi and should be further evaluated as a diagnostic test for melanocytic lesions that are now ambiguous. In addition, we show marked differences in the genetic make-up of melanomas that depend on anatomical location and sun-exposure pattern. These finding should motivate further studies to establish they are indicative of unique therapeutic opportunities for melanomas of different types.

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